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Preferential Inhibition of Ribonucleic Acid Synthesis by a New Thiosemicarbazone Possessing Antibacterial and Antiparasitic Properties

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We determined the influence of the azacycloheptane derivative (H) of a 2-acetylpyridine thiosemicarbazone on growth and macromolecular synthesis in *Escherichia coli* AT-9. Thiosemicarbazone H caused bacteriostasis and a primary inhibition of ribonucleic acid synthesis; secondary effects included inhibition of deoxyribonucleic acid and protein synthesis. Addition of copper or other transition elements was not necessary for these inhibitions.

Thiosemicarbazones containing highly varied substituents have been found to possess chemotherapeutic properties against a wide variety of microorganisms and diseases (7). These compounds, most frequently in chelation with transition elements, appear to inhibit the function of nucleic acids and related enzymes. Recently, the synthesis of a new class of thiosemicarbazones, the 2-acetylpyridines, was described, and screening studies revealed compounds of this group to inhibit the growth of bacteria, plasmodia, and leishmanial promastigotes in vitro (4-6; B. D. Hansen and A. D. Wolfe, unpublished data). An appreciable percentage of compounds possessed minimal inhibitory concentrations of 0.062 μg/ml or less against *Neisseria gonorrhoeae* and *N. meningitidis*, whereas suppressive effects against *Plasmodium berghei* in mice were observed at concentrations as low as 20 mg/kg. These extensive chemotherapeutic properties have led to the present studies on the mode of action of the azacycloheptane derivative (H) (Fig. 1) of this class, using *Escherichia coli*, *P. berghei*, and *Leishmania braziliensis* as test organisms. This communication describes the influence of thiosemicarbazone H on *E. coli*. Primary effects included bacteriostasis and inhibition of ribonucleic acid (RNA) synthesis, with secondary inhibition of deoxyribonucleic acid (DNA) and protein synthesis.

MATERIALS AND METHODS

The final specific activities, concentrations, and sources of isotopically labeled compounds were as follows: [2-^{14}C]uracil, 0.64 mCi/mmol, 0.04 mCi/liter; [1-*U*-^{14}C]phenylalanine, 0.53 mCi/mmol, 0.05 mCi/liter; [*G*-^{3}H]deoxyadenosine, 8.06 mCi/mmol, 0.25 mCi/liter (New England Nuclear Corp., Boston, Mass.); [*G*-^{3}H]adenine, 2.7 mCi/mmol, 0.2 mCi/liter (Amersham Corp., Arlington Heights, Ill.). L-Methionine was a product of Nutritional Biochemicals Corp., Cleveland, Ohio. Turbidimetric determinations were conducted in a model 250 spectrophotometer (Gilford Instruments Laboratories, Inc., Oberlin, Ohio); isotope incorporation was measured in a Mark III liquid scintillation spectrometer (Searle-Analytic Corp., Chicago, Ill.). Centrifugation was routinely performed in an RC2-B centrifuge, using an SS-34 rotor (Sorvall Instrument Co., Wilmington, Del.). Bacteria were grown and experiments were performed in a model 50 shaking water bath produced by GCA/Precision Scientific Co., Chicago, Ill. Nitrocellulose filters (type HA, 0.45 μm) were products of the Millipore Corp., Bedford, Mass. The liquid scintillation cocktail, Instabray, was purchased from Yorktown Laboratories, Hackensack, N.J., and dimethyl sulfoxide (DMSO) was from J. T. Baker Chemical Co., Phillipsburg, N.J. Thiosemicarbazone H was a gift of Daniel L. Klayman, Division of Medicinal Chemistry, Walter Reed Army Institute of Research, Washington, D.C.

All experiments used *E. coli* AT-9, Met$^-$, growing exponentially in M9 medium (13) supplemented with 20 μg of L-methionine per ml. Bacterial cultures were grown at 37°C and 80 oscillations per min, and growth was measured turbidimetrically at 540 nm. A 1-mg/ml solution of drug dissolved in DMSO was prepared immediately before use, and equal volumes of drug and DMSO were added to experimental and control cultures at appropriate times. Drug was always diluted 100-fold into experimental cultures, and 1% DMSO did not affect cell growth.

Synthesis of RNA, DNA, and protein was determined by measurement of the incorporation of the appropriate isotopically labeled compound into cold (4°C) trichloroacetic acid-insoluble fractions. Exponentially growing bacteria were adjusted to a turbidity of 0.05 at 540 nm; drug, drug solvent, and isotopically labeled compound were added; and 2.0-ml portions were withdrawn at time intervals. Portions were chilled by dilution with physiological saline at 4°C and centrifuged at 10,000 rpm for 10 min, and the pellets were washed and suspended three times in fresh physiological saline. Trichloroacetic acid (100%) was added to each suspension to bring the final concentration to

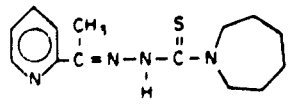
10%, and cells were incubated for 1 h at 4°C. Extracted suspensions were centrifuged as previously, the supernatants were withdrawn, and the pellets were washed free of unincorporated radioactivity. Residues were then either (i) solubilized in 2.0 ml of 100% formic acid, and a 1.0-ml portion was counted, or (ii) solubilized with 2.0 ml of 5% trichloroacetic acid for 1 h at 70°C. Then the extract and the insoluble residue were separated by centrifugation, the residue was resuspended in 5% trichloroacetic acid and collected on nitrocellulose filters, and both fractions were counted.

RESULTS

Thiosemicarbazone H was bacteriostatic rather than bactericidal for *E. coli* AT-9. Bacteria incubated with graded concentrations of H exhibited reduced linear growth, and colony count (not shown) indicated that viability remained constant.

Drug action varied with the culture medium. Use of brain heart infusion broth or yeast extract reduced inhibition. Preincubation of H in 2×10^{-4} M unbuffered glutathione or glutamic acid, but not cysteine, inactivated H and eliminated an absorption peak at approximately 305 nm. Drug preincubation in 10^{-3} M HCl also inactivated H.

The influence of H on growth was determined



1-Acycloheptanone 1 thiocarboxylic acid 2 [1 (2 pyridyl) ethylidene] hydrazide

FIG. 1. *Structure of thiosemicarbazone H.*

turbidimetrically and by comparison (Fig. 2) of macromolecular precursor incorporation rates in drug-free and drug-containing cultures. Incorporation of uracil was more severely inhibited than incorporation of either deoxyadenosine or phenylalanine. The data suggest RNA synthesis to be more sensitive to H than either DNA or protein synthesis, and conversion of the 60-min isotope incorporation data to percentage of inhibition depicted in the probit transform (Fig. 3) clearly documents this finding. Graded concentrations of H which caused 70 to 90% inhibition of RNA synthesis reduced DNA and protein synthesis from 40 to 70%. Inclusion of typical turbidimetric growth inhibition percentages for an identical time interval, and linear regression analysis, showed a single curve to relate inhibition of RNA synthesis to growth inhibition.

Since the data in Fig. 2 indicate the occurrence of a short lag before inhibition of deoxyadenosine incorporation, inhibition rates were determined after preincubation of bacteria with drug. The results of a typical experiment are shown in Fig. 4. Two drug-free subcultures were permitted to continue exponential growth, whereas H was added, to a final concentration of 3.6×10^{-5} M, to two additional subcultures. After 45 min of incubation, cultures were standardized to a turbidity of 0.20, [^{14}C]phenylalanine, in combination with either [^{3}H]uracil or [^{3}H]deoxyadenosine, was added to the subcultures, and incubation was continued. Portions were withdrawn at time intervals and processed to analyze inhibition of respective biosyntheses. The drug-containing cultures were equally inhibited, as indi-

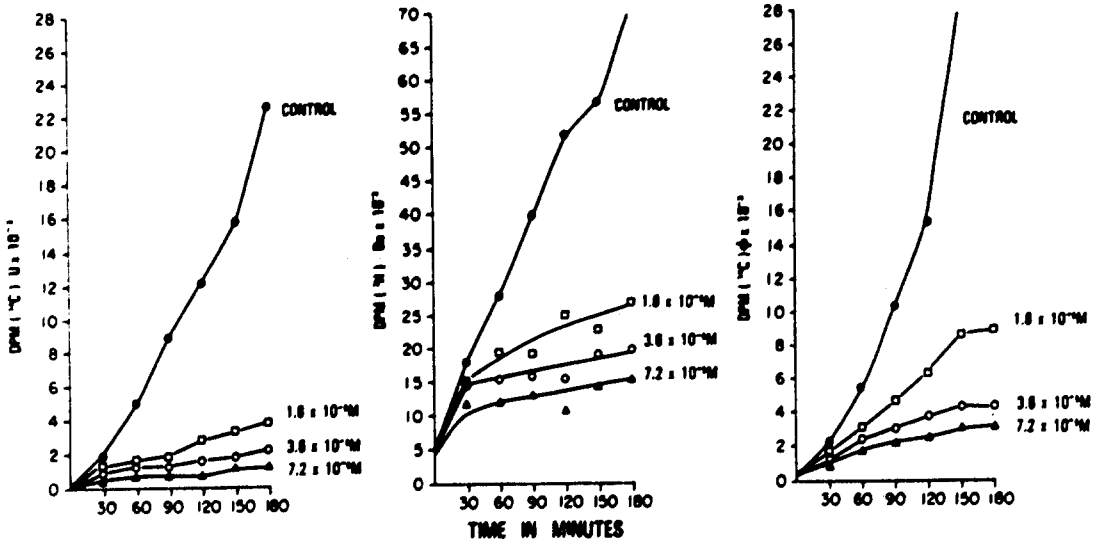


FIG. 2. *Incorporation of macromolecular precursors into bacteria in the absence of H and in the presence of the designated H concentrations. Ordinate: U, [^{14}C]uracil; Da, [^{3}H]deoxyadenosine; ϕ, [^{14}C]phenylalanine.*

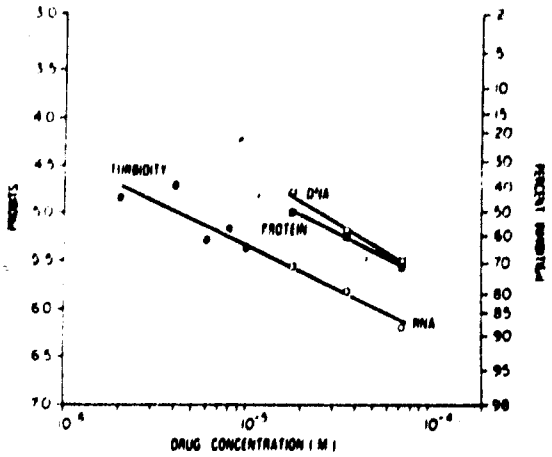


FIG. 3. *Probit transformation of 60-min data from Fig. 2 and from turbidimetric analysis of growth inhibition. The increase in turbidity was determined in drug-free and drug-containing cultures, and the percentage of inhibition during the initial 60-min incubation was calculated and plotted. Transposition of points to linear scales permitted least-squares analytical construction of the RNA-turbidity curve.*

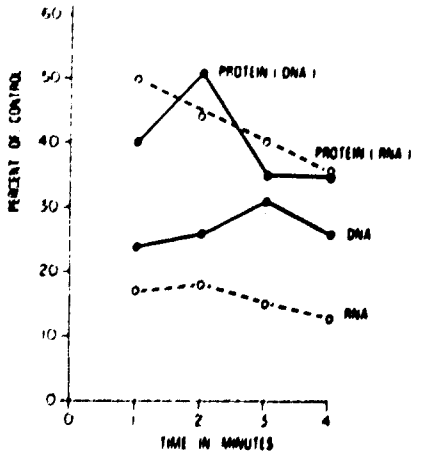


FIG. 4. *Percentage of incorporation, in comparison with drug-free cultures, of [^{14}C]phenylalanine, in combination with [^{3}H]uracil or [^{3}H]deoxyadenosine, in the absence and the presence of 3.6×10^{-5} M H. Bacteria were preincubated with the drug or with DMSO for 45 min, cultures were readjusted to a turbidity of 0.20, and isotopically labeled compounds were added. Culture samples of 5.0-ml volume were withdrawn at time intervals and treated as described in Materials and Methods, and hot trichloroacetic acid extracts and filter-entrapped radioactivity were counted. Protein (DNA) indicates [^{14}C]phenylalanine added with [^{3}H]deoxyadenosine; protein (RNA) indicates [^{14}C]phenylalanine added with [^{3}H]uracil; DNA indicates [^{3}H]deoxyadenosine added with [^{14}C]phenylalanine; RNA indicates [^{3}H]uracil added with [^{14}C]phenylalanine.*

cated by the suppression of protein synthesis, whereas RNA synthesis remained the most drug sensitive of the three processes. However, a more severe, time-dependent inhibition of DNA synthesis was now observed; preincubation to achieve more complete expression of drug action resulted in distinction between the sensitivity of those processes to H.

DISCUSSION

The present research has shown H to inhibit the growth of *E. coli* and has related growth inhibition to suppression of RNA synthesis. Inhibition of DNA and protein synthesis also occurred, and this may be a natural consequence of suppression of RNA replication. However, thiosemicarbazones exert multiple cellular influences (7), and the direct effects of H may be similarly extensive. For example, *N*-methyl isatin β-thiosemicarbazide equally inhibits *E. coli* RNA polymerase and DNA polymerase I (8). Polymerase I is not required (3) for growth, however, but possesses repair functions which were not tested in the present experiments. Isatin β-thiosemicarbazide inhibits late vaccinia virus protein synthesis by a mechanism which remains to be clarified (2). H may inhibit synthesis of RNA primer sequences required for DNA replication (17), but structurally specific pyridyl and isoquinoline thiosemicarbazones inhibit viral and tumor cell (1, 12) ribonucleotide reductases, enzymes which reduce ribonucleoside diphosphates to deoxyribonucleoside diphosphates in preparation for DNA synthesis. A similar effect of H may halt DNA synthesis after depletion of existing ribonucleoside diphosphate pools. Figure 2 indicates that DNA synthesis ceased abruptly after 30 min of incubation with H and suggests the potential occurrence of an independent inhibition of DNA synthesis. Preincubation experiments (Fig. 4) also support this concept and indicate the influence of H to occur on biosyntheses in the order: RNA > DNA > protein. Thus H inhibition of RNA replication may result in growth inhibition, but extensive investigation will be required to determine potential cellular effects.

In many instances, chelation with transition elements, notably copper, is necessary to thiosemicarbazone action. A fundamental mechanism has been proposed (11) to explain the influence of thiosemicarbazone-copper chelates; this mechanism involves cellular thiol group reduction, with consequent redox reactions and deposition of copper. However, the isatin β-thiosemicarbazides and H appear to act in the absence of an overt, exogenous transition element

requirement (2, 9, 10). *E. coli* RNA polymerase, DNA polymerase, and possibly Rous sarcoma virus RNA-dependent DNA polymerase contain zinc (14–16, 18), a transition element which may constitute a portion of the ultimate drug target. Whatever mechanism is involved, the 2-acetylpyridine thiosemicarbazones appear similar in action to other thiosemicarbazones, although the new compounds offer unusual chemotherapeutic promise in view of their potency and broad spectrum.

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